apd1$^+$, a Gene Required for Red Pigment Formation in *ade6* Mutants of *Schizosaccharomyces pombe*, Encodes an Enzyme Required for Glutathione Biosynthesis: A Role for Glutathione and a Glutathione-Conjugate Pump

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ABSTRACT

Mutants in the adenine biosynthetic pathway of yeasts (*ade1* and *ade2* of *Saccharomyces cerevisiae*, *ade6* and *ade7* of *Schizosaccharomyces pombe*) accumulate an intense red pigment in their vacuoles when grown under adenine-limiting conditions. The precise events that determine the formation of the pigment are however, still unknown. We have begun a genetic investigation into the nature and cause of pigmentation of *ade6* mutants of *S. pombe* and have discovered that one of these pigmentation defective mutants, *apd1* (adenine pigmentation defective), is a strict glutathione auxotroph. The gene *apd1*$^+$ was found to encode the first enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase, *gcs1*$^+$. This gene when expressed in the mutant could confer both glutathione prototrophy and the characteristic red pigmentation, and disruption of the gene led to a loss in both phenotypes. Supplementation of glutathione in the medium, however, could only restore growth but not the pigmentation because the cells were unable to achieve sufficient intracellular levels of glutathione. Disruption of the second enzyme in glutathione biosynthesis, glutathione synthetase, *gsh2*$^+$, also led to glutathione auxotrophy, but only a partial defect in pigment formation. A reevaluation of the major amino acids previously reported to be present in the pigment indicated that the pigment is probably a glutathione conjugate. The ability of vanadate to inhibit pigment formation indicated that the conjugate was transported into the vacuole through a glutathione-conjugate pump. This was further confirmed using strains of *S. cerevisiae* bearing disruptions in the recently identified glutathione-conjugate pump, *YCF1*, where a significant reduction in pigment formation was observed. The pump of *S. pombe* is distinct from the previously identified vacuolar pump, hmt1p, for transporting cadystin peptides into vacuoles of *S. pombe*.

THE intense red pigment that accumulates in certain adenine biosynthetic mutants of yeasts forms a simple and easily detectable phenotype that has been exploited since the very early days of yeast genetics (ROMAN 1956). These mutants are the *ade2* and *ade1* mutants of *Saccharomyces cerevisiae* and the *ade6* and *ade7* mutants of *Schizosaccharomyces pombe*. Equivalent mutants have also been identified in other yeasts (KLEIN and FAVREAU 1988; ZONNEVELD and VAN DER ZANDEN 1995). Defects in the *ADE2* gene of *S. cerevisiae* (equivalent to the *ade6*$^+$ gene of *S. pombe*) lead to accumulation of the intermediate phosphoribosylaminoimidazole (AIR), while a defect in the *ADE1* gene of *S. cerevisiae* (equivalent to *ade7*$^+$ in *S. pombe*), which encodes an enzyme in the subsequent step in the adenine biosynthetic pathway, accumulates the intermediate phosphoribosylaminoimidazole carboxylate (CAIR) (FISCHER 1969; reviewed in JONES and FINK 1982). These intermediates are only accumulated when the mutant cells are grown in adenine-limiting medium since the biosynthetic pathway is repressed during growth in adenine-sufficient conditions. Both the intermediates, AIR and CAIR, are not colored and it is only subsequent events after the accumulation of these intermediates that lead to the distinctive pigmentation in these mutants. Numerous physiological and environmental factors are known to influence the pigmentation. Growth temperatures, aeration, glucose concentrations, and the presence of different amounts of amino acids and purines in the medium are a few of the factors that influence the level of pigmentation (DORFMAN 1969). Petite mutants, on the other hand, are almost completely devoid of pigmentation and, indeed, this has been a characteristic means of identification of petite mutations. Despite this information, and the extensive and widespread use of these mutants even today, the precise events that are actually responsible for the formation of the pigment are still unknown. Structural analysis of the pigment has been elusive, and the limited structural analysis that has been carried out indicates it to be a conjugate of the adenine biosynthetic intermediates, AIR and CAIR, with certain amino acids followed by oxidative condensation and polymerization that leads to this characteristic color (SMIRNOV *et al.* 1967). The purification and characterization of the pigment has been complicated

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by the heterogenous nature of the compound and its propensity to undergo oxidation and polymerization (SMIRNOV *et al.* 1967; our unpublished results). The pigment itself has been observed to accumulate in the vacuole (WEISMAN *et al.* 1987), and mutants defective in vacuolar biogenisis lack this characteristic pigmentation even when grown in adenine-limiting medium (WADA *et al.* 1992), clearly implicating the vacuole also in these processes. To investigate these events, and find some unifying picture among these pieces of the puzzle, we decided to take a genetic approach to the problem and sought to isolate mutants of *S. pombe* that were defective in pigment formation. We report here our investigations on one of these mutants, *apd1*. These mutants were found to be strict glutathione auxotrophs, and cloning the gene for *apd1*$^+$ revealed it to be the first enzyme in glutathione biosynthesis. Our results demonstrate for the first time a role for glutathione in the red pigment formation in these yeasts. In addition, our results also demonstrate the existence of a glutathione-mediated detoxification pathway for the removal of endogenously derived metabolites from the cytosol into the vacuole.

MATERIALS AND METHODS

Yeast strains, growth conditions and plasmids: The wild-type *Schizosaccharomyces pombe* (*h*$^-$ *ura4Δ18 ade6-210*) strain used for most of these studies and to generate the mutants described in this work, as well as *S. pombe* strain *h*$^+$ *ura1-1*, was obtained at the Fission Yeast Course, Cold Spring Harbor Laboratory. *S. pombe h*$^+$ *ura4Δ-18 ade6-210*, *S. pombe h*$^-$ *ura4Δ-18 ade6-210 apd1-1*, *S. pombe h*$^-$ *ura4Δ-18 ade6-210 gcs1Δ::ura4*$^+$, *S. pombe h*$^+$ *ura4Δ-18 ade6-210 gsh2Δ::ura4*$^+$ and *S. pombe h*$^+$ *ura4Δ-18 ade6-210 hmt1Δ::ura4*$^+$ were constructed in this laboratory. *S. cerevisiae* (*YCF1*) *MAT***a** *ura3-52 leu2-Δ lys2-801 ade2-101 his3-Δ200 trp1-Δ63* (YPH4999; SIKORSKI and HIETER 1989) was obtained from Dr. KARL KUCHLER and *S. cerevisiae* (*ycf1Δ*) *MAT***a** *urs3-52 leu2-Δ1 lys2-801 ade2-101 his3-Δ200 trp1-Δ63 ycf1Δ::KanMX2* was constructed in this laboratory. *S. pombe* was routinely grown in YES medium that contained 0.5% yeast extract, 3% glucose, and supplements (50 mg/liter uracil and 50 mg/liter adenine). Adenine-limiting medium contained adenine at a concentration of 10 mg/liter. Minimal media growth was in Edinburghs Minimal Medium (EMM) (ALFA *et al.* 1992) with supplements added as described, except that we routinely used sodium glutamate at 0.5% instead of ammonium sulphate as a source of nitrogen. Agar was added at a concentration of 1.5% in plates. Malt extract agar medium contained 3.0% malt extract and 2% agar. *S. pombe* cells were grown routinely at 30°. $CdCl_2$ plates were prepared by addition of 100 μM cadmium chloride to YES medium after autoclaving. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) plates were prepared by addition of MNNG to YES plates to give a final concentration of 40 μg/ml, and was added while pouring the plates. Minimal plates containing glutathione were supplemented with reduced glutathione at concentrations of 0.5 mM. In vanadate-containing plates, sodium orthovanadate was added at concentrations of 80 to 200 μg/ml to YES plates or broth containing limiting amount of adenine. *S. cerevisiae* were grown in YPD medium supplemented with tryptophan.

All chemicals were of analytical reagent grade and were obtained from Sigma Chemical Co. or from Sisco Research Laboratories, Mumbai, India. Media components were from DIFCO. Oligo nucleotides were obtained from Ransom Hill Bioscience.

Plasmid pAS1 (V1142), which contained the *ADE6* and *URA3* genes on an *S. pombe* plasmid, was a gift of Dr. GERALD SMITH. The *S. pombe* genomic libraries pSP1 and pSP2 were provided by Dr. ANTHONY CARR.

Mutagenesis and isolation of mutants: Overnight grown cells of *S. pombe* were reinoculated into fresh YES medium and allowed to grow to an OD_{600} = 0.5. The cells were harvested, washed twice with sterile water and finally suspended in 0.1 volume of minimal medium. Ethyl methyl sulfonate was added at a final concentration of 2% and the cells were incubated at 30°. At different time intervals aliquots were taken, washed and plated onto YES medium containing limiting adenine. At conditions that allowed 10% survival, putative mutants were picked up and purified by restreaking for single colonies on the same medium.

Genetic analysis: *Random spore analysis:* Random spore analysis was used for strain construction, backcrossing and complementation analysis. Two yeast strains in question of opposite mating type were freshly grown on YES plates. The strains were mixed on MEA plates and incubated at room temperature for 2 days. Ascus formation was examined under the light microscope, and after sufficient asci could be detected, the cells were treated with glusulase (0.5%) overnight at 37°; (ALFA *et al.* 1992) after appropriate dilutions, the spores were allowed to germinate on YES plates. To determine if the glusulase was indeed effective in killing all diploids and haploids except the spores, we also periodically carried out control experiments where we carried out the cross with strains of the same mating type. Virtually no colonies were observed even at low dilutions.

Complementation analysis: Complementation analysis by diploid formation through intragenic complementation of the *ade6* alleles was hampered by the fact that the different *ade6* alleles caused different degrees of pigmentation. Therefore we carried out complementation analysis through random spore analysis as described above. The mutants that were in an *h*$^-$ *ade6-210 ura4Δ18* were crossed with mutants that were *h*$^+$ *ade6-210 ura4Δ18* and spore colonies were examined for their ratio of white to red color to determine the complementation groups. The ratio of red to white spore colonies was expected to be 1:3 in the case of complementation. In the case of noncomplementation the ratio of red to white spore colonies would be 0:4.

Backcrossing of apd1-1: apd1-1 (*h*$^-$ *ura4Δ18 ade6-210*) was backcrossed twice with wild-type strains. In the first backcross it was crossed to *h*$^+$ *ura1-1*. After random spore analysis, spore clones that were *h*$^+$ *ade6-210 apd1*(ura$^+$) were picked, as these were clearly spores that arose as a result of the cross, and were crossed a second time with strains that were *h*$^-$ *ura41Δ18 ade6-210*. Spore clones from random spore analysis that were *h*$^-$ *ura4Δ-18 ade6-210 apd1* were a result of this cross and were picked and kept for further studies.

Estimation of glutathione in cell extracts: Glutathione was estimated by the 5,5′dithiobis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase coupled assay essentially as described earlier (ANDERSON 1985). Cells were harvested at the desired time intervals, washed twice with water, resuspended in 0.4 ml of 5% sulphosalicylic acid, mixed with an equal volume of glass beads, and broken by vigorous bead beating in a bead beater (Biospec) at 4° for a total of 6 min with intermittent pauses. The extracts were spun down in a microfuge to remove the glass beads and also the cell debris and protein precipitate. The supernatant was assayed to determine the amount of glutathione. The estimation of the glutathione

content in YES medium was also estimated using this method by taking an aliquot of YES medium, which contains 0.5% yeast extract.

Spectra of pigment: Red pigment formation was estimated by examination of the spectra of extracts made in 5% sulphosalicylic acid. An equal amount of cells grown in YES medium containing limiting adenine were harvested at OD_{600} = 3.0, washed with water and broken in a bead beater with glass beads as described above. For blanks we used the extracts made from *apd1-1* mutants. To determine the relative concentrations of the pigment we took the absorbances at the peak value, 530 nm.

Transformation of *S. pombe*: *S. pombe* cells were grown to early exponential phase (OD_{600} = 0.5), harvested, washed once with sterile water, and finally suspended in 0.5–1 ml of 0.1 M lithium acetate solution pH 4.9. To 0.1-ml aliquots of these cells 50 μg of salmon sperm carrier DNA was added along with the plasmid/DNA fragment and incubated at room temperature for 10 min. One half milliliter of 50% PEG (in 0.1 M lithium acetate, pH 4.9) was added and incubated at 30° for 45 min. The whole mixture was then incubated at 46° for another 25 min and transferred to room temperature for at least 10 min. The transformed cells were collected by centrifugation, resuspended in sterile water and immediately plated on the selection plates.

Isolation of genomic DNA and plasmid DNA from yeast: Plasmid DNA and genomic DNA were isolated from *S. pombe* using the glass bead lysis methods described for *S. cerevisiae* (STRATHERN and HIGGINS 1991; KAISER *et al.* 1994).

Disruption of *gcs1*$^+$: Disruption of the *gcs1*$^+$ gene was carried out by using the one-step PCR disruption method first described for *S. cerevisiae* (BAUDIN *et al.* 1993) using oligonucleotides for the *S. pombe ura4*$^+$ gene that had flanking residues of *gcs1*$^+$. PCR was carried out with a pBSK plasmid containing the *S. pombe ura4*$^+$ gene as template. The 1.7-kb PCR product obtained was purified and used for transformations into haploid *S. pombe*, selecting for the uracil marker in medium supplemented with glutathione. Disruptions in *S. pombe* occur at a low but definite frequency with 40-bp flanking residues (KAUR, INGAVALE and BACHHAWAT, unpublished results), and we were able to obtain disruptants, *gcs1*Δ*::ura4*$^+$ that carried a full disruption by this method (Figure 3). The oligonucleotides used for making this disruptant were gs1del1 and gs1del2, 5′-TTGCACATGTATGATACTTATATATCTAAAAAGCAAGATGCTACAAATCCCACTGGC-3′ and 5′-CGGCATGTACAGATTCAAATTGAAGGATAGAATTGCACGAGAAAGCTTGTGATATTGACG-3′, respectively. Disruptions were confirmed by PCR using the oligonucleotides, gs1fp and gs1rp whose sequences are 5′-GAAGTGTGCAAACCTTACTAAACCG-3′ and 5′-TATTCCACTCAGAATTCATCTGC-3′, respectively. The underlined regions correspond to *ura4*$^+$ sequences.

Disruption of *hmt1*$^+$ and *gsh2*$^+$: To disrupt the *hmt1*$^+$ gene we employed the one-step PCR disruption method described above. The oligonucleotides used for the PCR disruption were hmtdel1 and hmtdel2, 5′-TGGATAATTGTACTTATCTCATGCGCAGATTCTAAAGCTCTTGCTACAAATCCCACTGGC-3′ and 5′-AAAGCCGAATGTTACTCTATAAGCACTTAATAGACAAGCAAAGCTTGTGATATTGACG-3′, respectively. Transformation with the PCR product was carried out in haploid *S. pombe* cells (*h*$^-$ *ade6-210 ura4-D18*). The oligonucleotides, hmtfp, 5′-GCAGTTCCAAATTGGTGGCCCTGT-3′ and hmtrp, 5′-AAGAGGCTGCTGTAGCTGAATCATG-3′, were used to confirm disruption at the *hmt1* locus by PCR.

The oligonucleotides used for the PCR disruption of *gsh2* were gs2del1 and gs2del2, 5′ACGGACCAACGCACCCTGGAATATGAGTTGCTTAATCGATTCGCTACAAATCCCACTGGC-3, and 5′-GATGTTCCAAACGATCGTACCTAAAATACCAATTTCGTCGACAAGCTTGTGATATTGACG-3′, respectively. The oligonucleotides, gs2fp, 5′-GGTAATAGTAGTTTGACGCTGTG-3′ and gs2rp, 5′-CAACATCTTCACGTTTGCATCAC-3′, were used to confirm whether disruption had occurred at the *gsh2* locus. The underlined regions correspond to *ura4*$^+$ sequences.

Disruption of *YCF1*: *YCF1* was disrupted using the one-step PCR method using the Kanamycin modules (WACH *et al.* 1994). Transformants were initially selected on 200 μg/ml G418 and subsequently checked for cadmium sensitivity. The oligonucleotides used in the disruption were ycfdel1, 5′-TGGGCCTGCAAGCTCTGTAGATCTCCTGAAGGGTTTGGACAGCTGAAGCTTCGTACG-3′ and ycfdel2, 5′-TCCTGTCTCTCACTGTTATAGTCTGTGACGAACTTGATTGCATAGGCCACTAGTGGATC-3′. The underlined regions correspond to the KanMX2 module.

RESULTS

Isolation and characterization of *apd* (adenine pigmentation defective) mutants: *S. pombe* strains carrying an *ade6-210* mutation accumulate an intense red pigment when grown under adenine-limiting conditions. This strain was therefore used as the parent strain to isolate mutants that were completely defective in pigmentation when grown in YES medium containing 10 mg/liter added adenine (as opposed to 50 mg/liter for adenine-sufficient growth). Thirty such *apd* (adenine pigmentation defective) mutants were isolated. To eliminate those mutants where reversion or suppression of the *ade6-210* phenotype may have occurred, we checked these mutants for growth on medium lacking adenine. Six of the mutants showed relatively better growth than the parent strain on minimal medium lacking adenine and probably contained either reversions or suppressor mutations. These mutants were therefore not pursued further. Mutations upstream of *ade6*$^+$ in the adenine biosynthetic pathway would also lead to a similar pigmentation defective phenotype. To identify these mutants, we transformed the *apd* mutants with a plasmid, pAS1, containing *ade6*$^+$ and *URA3* (PONTICELLI *et al.* 1988). The transformants were selected on minimal plates containing adenine but lacking uracil and were subsequently patched on minimal plates lacking adenine. Mutants that contained this *ade6*$^+$ plasmid but were still unable to grow on medium lacking adenine probably had a second mutation in the adenine biosynthetic pathway upstream of *ade6*$^+$ and these also were not pursued further. In fact, 19 mutants appeared to carry a second mutation in the adenine biosynthetic pathway. Only two mutants could not be checked by this procedure initially as they failed to grow in minimal medium supplemented with only adenine and uracil. These are described in more detail below. Complementation analysis of the remaining five mutants, including the two auxotrophs, were carried out by random spore analysis and placed into four complementation groups (*apd1–apd4*).

***apd1* mutants are glutathione auxotrophs:** Two of the mutants that failed to grow on minimal medium even

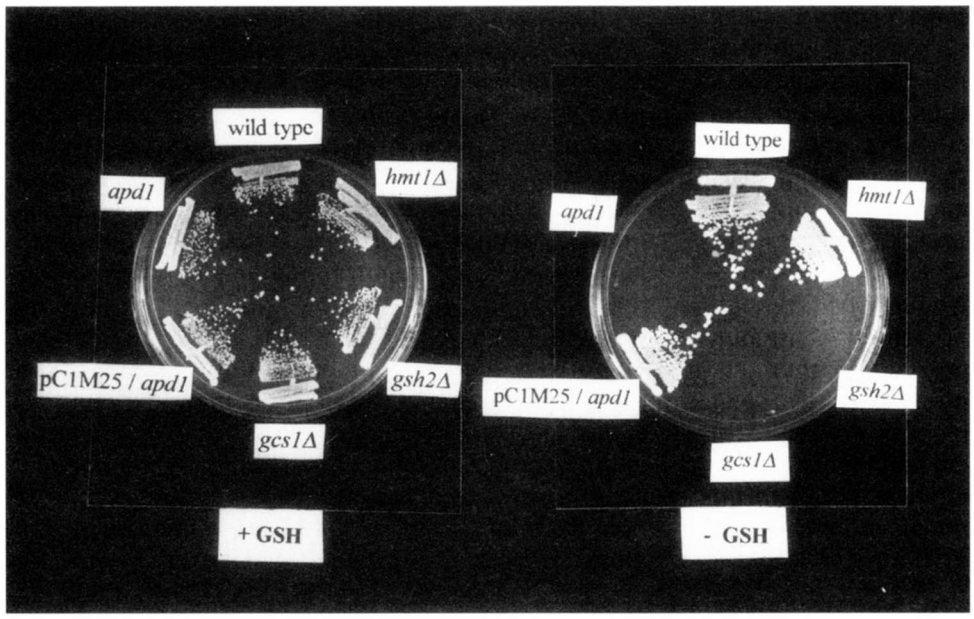

FIGURE 1.—Growth of *S. pombe* wild-type and mutant strains on minimal plates and minimal plates supplemented with glutathione. *S. pombe* wild-type cells, *apd1-1*, *gcs1Δ::ura4⁺*, *gsh2Δ::ura4⁺* and pC1M25/*apd1-1* and *hmt1Δ::ura4⁺* streaked onto EMM (−GSH) and EMM containing 0.5 mM of reduced glutathione (+GSH). *gcs1⁺*, which encodes γ-glutamylcysteine synthetase, *gcs2⁺*, which encodes glutathione synthetase, and *hmt1⁺*, which encodes a vacuolar heavy metal transporter, are discussed in detail later in the text.

when mixtures of all amino acids, vitamins and nucleotides were added, either separately or together, also failed to sporulate when crossed with each other and were tentatively placed in the same complementation group (*apd1*). Backcrossing of *apd1-1* twice with wild-type strains (as described in MATERIALS AND METHODS) indicated that the lack of pigmentation of these mutants cosegregated with the auxotrophy. During analysis of the secondary phenotypes of these four complementation groups, we also discovered that the *apd1* complementation group showed sensitivity to increased concentrations of $CdCl_2$ in the medium but not to NaCl, $CaCl_2$ or $MgCl_2$. This suggested that it was probably not a vacuolar biogenesis mutant as defects in vacuolar biogenesis lead to pleiotropic ion sensitivity. Mutations in the glutathione biosynthetic pathway, on the other hand, are known to be specifically sensitive to cadmium and other heavy metals (GLAESER *et al.* 1991) so we tried adding glutathione to the minimal medium. Interestingly, the *apd1* mutants grew very well in minimal medium supplemented with glutathione and were clearly glutathione auxotrophs (Figure 1). The ability of *apd1* mutants to grow in YES medium suggested the presence of glutathione in yeast extracts. To confirm this, we determined the glutathione content in yeast extract, and found that yeast extract indeed contained significant levels of glutathione [~2% (w/w) glutathione]. The concentration of glutathione in the YES medium that we use (which contains 0.5% yeast extract) would therefore be ~0.25 mM, a concentration sufficient to support growth of these mutant cells in this medium.

Growth experiments in minimal medium and with glutathione supplemented at a concentration of 0.5 mM, confirmed that glutathione was, in fact, essential for growth. In media that lacked any glutathione the cells failed to grow beyond a single doubling (data not shown). We also found a complete inhibition of spore formation, which explains why we were initially unable to obtain spores in the cross between the two auxotrophic mutants. Subsequent experiments where we supplemented the sporulation plates with glutathione allowed the formation of a small percentage of spores, and using these conditions we could ascertain that these mutants were indeed in the same complementation group.

Although the mutants grew fairly well in YES medium and even in minimal medium supplemented with glutathione, they failed to develop the significant levels of pigmentation as compared to the wild-type strain, even when supplemented with 4 mM glutathione (data not shown). This was surprising since our genetic experiments indicated that the two phenotypes cosegregated. We therefore considered it essential to clone the gene that could complement the defect in pigmentation.

***apd1⁺* is *gcs1⁺* γ-glutamylcysteine synthetase, the first enzyme in glutathione biosynthesis:** To identify the gene, mutations, in which, resulted in a defect in pigment formation and also caused glutathione auxotrophy, we transformed *apd1-1* with a *S. pombe* genomic library (BARBET *et al.* 1992) and selected transformants that showed wild-type levels of pigmentation on adenine-limiting minimal plates that lacked both added uracil and glutathione. A total of three transformants were obtained. Plasmid was rescued from these yeast transformants and restriction digested to identify unique plasmids. Two different plasmids were obtained that also appeared to have overlapping and matching digests. These were retransformed into *apd1-1*. The transformants were not only glutathione prototrophs (Figure 1) but also had the characteristic red pigmentation when grown in adenine-limiting medium (Figure 2) and conferred wild-type resistance to cadmium (data

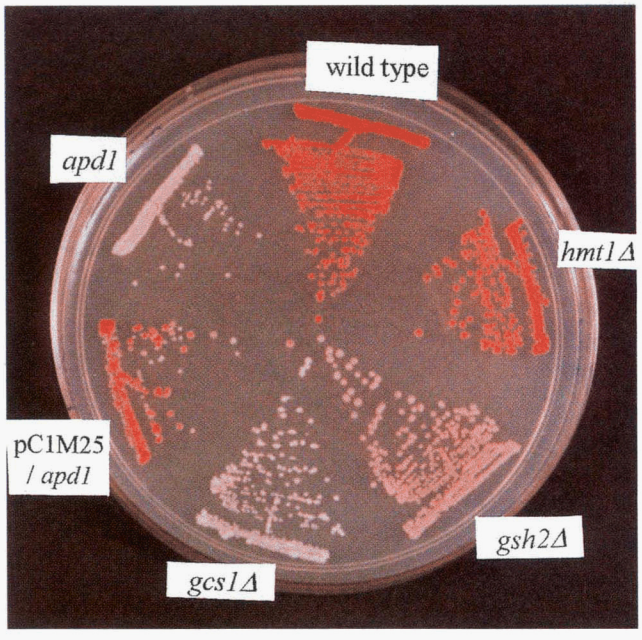

FIGURE 2.—Pigment formation in *S. pombe* wild-type and mutant strains on YES medium containing limiting amount of adenine. (a) *S. pombe* wild-type cells, (b) *hmt1Δ::ura4*$^+$, (c) *gsh2Δ::ura4*$^+$, (d) *gcs1Δ::ura4*$^+$, (e) pC1M25/*apd1-1* and (f) *apd1-1* were streaked onto YES medium containing limiting amount of adenine (10 mg/liter).

not shown). One of these plasmids, pC1M25, was used in all future experiments. Since we had suspected from our auxotrophy studies that either the first or second enzyme of glutathione biosynthesis (*gcs1*$^+$ or *gsh2*$^+$) may be *apd1*$^+$, we compared the restriction fragments generated from pC1M25 (Figure 3) with the published maps of these genes (MUTOH *et al.* 1991; COBLENZ and WOLFE, 1995; MUTOH *et al.* 1995). Restriction mapping strongly indicated that *apd1*$^+$ was probably the same as *gcs1*$^+$. This was confirmed by doing a PCR with the plasmid pC1M25 as template, using oligonucleotides specific to the two different genes, *gcs1*$^+$ and *gsh2*$^+$. A PCR product of the expected size was obtained using the oligonucleotides designed for *gcs1*$^+$ and the PCR product gave the expected sizes after restriction digestions. This confirmed that *apd1*$^+$ was indeed *gcs1*$^+$, which encodes γ-glutamylcysteine synthetase, the first enzyme in glutathione biosynthesis. While mutation in this gene was clearly responsible for the auxotrophy, we still needed to confirm that it was this gene (and not a closely linked gene on the same fragment) that was responsible for the pigmentation phenotype. We therefore disrupted this gene according to the strategy shown in Figure 3 and described in MATERIALS AND METHODS. Transformants were initially selected on minimal medium lacking uracil but containing adenine and glutathione and then subsequently checked on medium lacking glutathione. These were also confirmed by PCR (data not shown). We observed that disruption of *gcs1*$^+$ led not only to glutathione auxotrophy (Figure 1), cadmium sensitivity and MNNG resistance (data not shown) that was characteristic of a disruption in *gcs1* (COBLENZ and WOLFE 1995; MUTOH *et al.* 1995), but also to a defect in pigment formation (Figure 2) indicating that the same gene was responsible for all these phenotypes.

Since the presence of the *apd1*$^+$ (*gcs1*$^+$) clone in the *apd1* mutants could restore both the distinctive red pigmentation and the prototrophy, whereas supplementation of glutathione in plates conferred the ability to grow in minimal medium but did not restore the pigmentation phenotype, we wished to examine if this

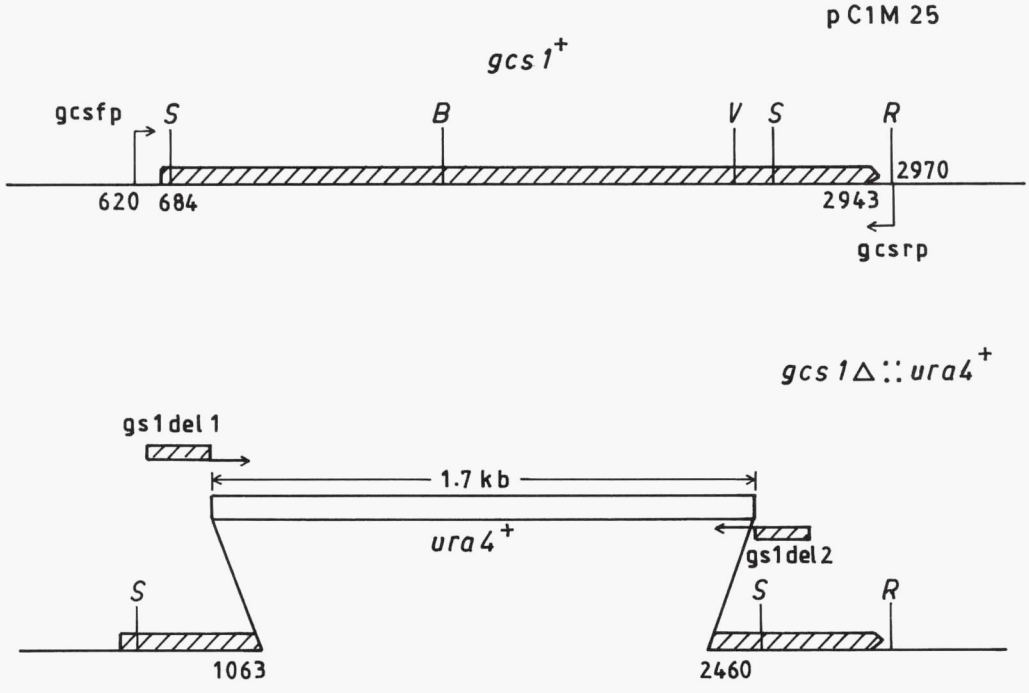

FIGURE 3.—Restriction map and strategy for disruption of *apd1*$^+$ (*gcs1*$^+$) gene. Partial restriction map indicating relevant restriction sites of the plasmid pC1M25, which contains the *apd1*$^+$(*gcs1*$^+$) gene and strategy for construction of deletion disruption using the one-step PCR method described in MATERIALS AND METHODS. The shaded region represents the *gcs1*$^+$ ORF. *S*, *Sca*I; *R*, *Eco*RI, *V*, *Eco*RV; *B*, *Bam*HI. The maps have not been drawn to scale.

FIGURE 4.—Comparison of intracellular glutathione levels of wild-type, *gcs1*Δ and *gsh2*Δ cells. *S. pombe* wild-type, *gcs1*Δ::*ura4*$^+$ and *gsh2*Δ::*ura4*$^+$ cells were grown in YES medium overnight and reinoculated in YES to an initial OD_{600} of ~0.1–0.2. Equal number of cells were harvested at midexponential phase (approximate OD_{600} = 1.5) and early stationary phase of growth (approximate OD_{600} = 4.5). Cells were washed twice with water and extracts were made in 5% sulphosalicylic acid as described in MATERIALS AND METHODS. An aliquot was used for estimation of glutathione using the DTNB-glutathione reductase assays with glutathione standards. At midexponential phase the wild-type value (100%) was 2.9 nmol GSH/10^7 cells; at stationary phase the wild-type value (100%) was 3.2 nmol GSH/10^7 cells. The values given are of a representative experiment.

might be a reflection of the intracellular glutathione concentrations in these cells. To address this question we determined the intracellular concentrations of glutathione of *gcs1*Δ strains and wild-type cells in YES medium at different stages of growth. Despite the presence of glutathione in the medium, at midexponential phase of growth the glutathione level in the *gcs1*Δ cells was only 20% that of the wild-type cells and at the late exponential/early stationary phase of growth, when pigment accumulation commenced, the intracellular glutathione levels of *gcs1*Δ fell further to 10% of the level seen in wild-type strains grown under the same conditions (Figure 4). Since the pigmentation occurred at the late stages of growth when the small amount of adenine added to the medium to allow growth was eventually depleted, the cells were greatly reduced in their intracellular glutathione levels. While these levels permitted slow growth, they clearly did not permit formation of the pigment.

Disruption of glutathione synthetase (*gsh2*$^+$), the second enzyme in glutathione biosynthesis leads to a partial defect in pigment formation: *gcs1*$^+$, γ-glutamylcysteine synthetase, is the first enzyme and the rate-limiting step in glutathione biosynthesis. The second enzyme, glutathione synthetase, *gsh2*$^+$, ligates γ-glutamylcysteine to glycine to form glutathione. Disruption of the second enzyme would, therefore, also lead to a deficiency in intracellular glutathione although it would lead to accumulation of the precursor γ-glutamylcysteine. This intermediate also contains the active sulphydryl, and we wished to examine whether disruption of *gsh2*$^+$ would also lead to a defect in pigment formation, which would indicate a requirement for glutathione alone that could not be replaced by its intermediate. We carried out the disruption of *gsh2*$^+$ according to the strategy described in MATERIALS AND METHODS. Ura$^+$ transformants were selected on minimal medium lacking uracil but containing glutathione. The transformants were screened for cadmium sensitivity. One of the sensitive mutants was confirmed to carry a disruption in the *gsh2*$^+$ gene by PCR (data not shown). The *gsh2*Δ::*ura4*$^+$ disruptants so obtained were checked for growth on minimal medium lacking glutathione. No growth could be observed in these plates but supplementation of the plates with 0.5 mM glutathione led to very good growth (Figure 1). This indicated that the intermediate γ-glutamylcysteine that accumulated in *gsh2*Δ strains could not replace the essential functions of glutathione. When these cells were streaked onto YES medium containing limiting amounts of adenine, however, we observed that the cells developed partial pigmentation as seen on plates (Figure 2), and as estimated spectrophotometrically by estimating the absorbance of the pigment at 530 nm (data not shown). Prolonged incubation of the plates led to increased levels of pigmentation. The results were surprising since the *gsh2*Δ cells also had low levels of intracellular glutathione similar to *gcs1*Δ cells (Figure 4). These findings indicated that although the intermediate γ-glutamylcysteine could not replace the essential growth functions carried out by glutathione, it could replace partially the role of glutathione in the formation of the pigment.

Pigment formation is vanadate sensitive, evidence for a glutathione-conjugate pump: Glutathione is known to play many roles within the cell, including maintainance of the redox status of the cells, which promotes the proper folding of proteins (MEISTER, 1988; PENNINCKX and ELSKENS 1993). Therefore it was not clear to us whether the absence of glutathione led to inactivation in enzymes required for the formation of the pigment, or alternately, whether glutathione was directly involved in pigment formation through the formation of a glutathione conjugate.

A partial structural analysis of the red pigment formed in *ade2* mutants of *S. cerevisiae* (SMIRNOV *et al.* 1967) had revealed that the pigment primarily contained glutamic acid, glycine, cysteine and aspartic acid as the prominent amino acids apart from the intermediate AIR. Although the authors had not concluded conjugation of glutathione to the intermediate in this report, the presence of glutamic acid, glycine and cysteine

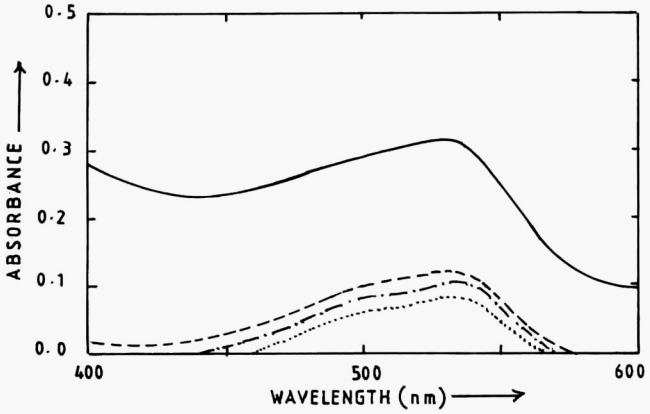

FIGURE 5.—Spectra of extracts made from cells grow in presence and absence of vanadate. Spectra from extracts of *S. pombe* parental strain (*h⁻ ura4-Δ18 ade6-210*) cultures grown in the presence and in increasing concentrations of sodium orthovanadate. Equal number of cells were harvested at the same phase of growth (approximate OD_{600} = 3.0), washed with water twice and extracts made in 5% sulphosalicylic acid as described in MATERIALS AND METHODS. Spectra were taken in a Shimadzu spectrophotometer with extracts of *apd1-1* used as blanks. *S. pombe* parent (——), 80 μg/ml vanadate (---), 160 μg/ml vanadate (-·-·-) 200 μg/ml vanadate (· · ·). Peak absorbances were at 530 nm.

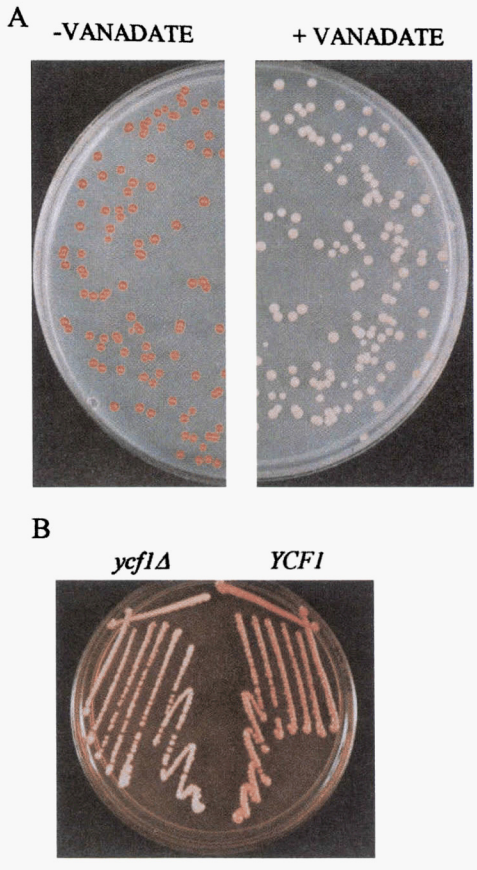

FIGURE 6.—(A) Effect of vanadate on pigment formation in *S. pombe*. *S. pombe* wild-type (*h⁻ ura4-Δ18 ade6-210*) cells were grown in YES broth, diluted appropriately and plated onto YES plates containing limiting adenine (10 mg/liter) (− vanadate) and YES plates containing limiting amount of adenine (10 mg/liter) and sodium orthovanadate (200 μg/ml) (+ vanadate). (B) Comparitive pigment formation of *S. cerevisiae YCF1* and *ycf1Δ* strains in YPD plates. *S. cerevisiae* strains, *ycf1Δ* and *YCF1*, were streaked onto YPD medium lacking any added adenine.

strongly suggested to us, in the light of our findings described in this paper, the conjugation of glutathione (γ-glutamylcysteinylglycine) in the formation of this pigment.

If indeed the pigment was, as the structural data suggested, a glutathione conjugate, it followed that there should exist a glutathione-conjugate pump to transport the conjugate into the vacuole, the organelle where it ultimately accumulates. An *in vitro* system was not available for us to test this in isolated vacuoles since we did not know the nature of the conjugation of glutathione to the intermediate. However, as an alternate, we sought to examine whether vanadate, a known inhibitor of glutathione pumps (PIKULA *et al.* 1994), could inhibit pigment formation in *S. pombe*. Vanadate is an inhibitor of many processes within the cell, but at low concentrations it is a specific inhibitor of glutathione-conjugate pumps. To investigate this, we examined accumulation of pigment in yeast strains that were grown in limiting adenine medium and with different concentrations of vanadate ranging from 80 to 200 μg/ml. Even at the lower concentration ranges of vanadate there was a significant drop in pigment formation as seen in plates and as determined spectrophotometrically (Figure 5). And at concentrations of vanadate of 200 μg/ml, the cells accumulated significantly lower levels of pigment even on prolonged incubation on plates (Figure 6A). Growth rates in medium that contained 200 μg/ml of vanadate were only slightly retarded. To eliminate the possibility that glutathione biosynthesis itself might be somehow affected in the presence of vanadate, we also estimated the intracellular glutathione content of cells grown in the presence of vanadate. However, no significant difference could be observed in glutathione levels (data not shown) further substantiating that the inhibition in the presence of low concentrations of vanadate was a specific consequence of the inhibition of a vanadate-sensitive glutahione-conjugate pump.

Disruption of the vacuolar *hmt1* pump does not affect pigment formation: Fission yeast vacuoles have been shown to contain a vacuolar pump, hmt1p (ORTIZ *et al.* 1992), that is involved in transporting cadmium complexed to cadystin into the vacuoles. Cadystins (phytochelatins) are low molecular weight peptides that have the structure $(\gamma\text{-glu-cys})_{2-11}$-gly. As they contain a glutathione moiety on the carboxy-terminal end of the molecule (MURASUGI *et al.* 1981; GRILL 1985), and as this pump was sensitive to vanadate (ORTIZ *et al.* 1995), we decided to examine whether in the case of the glutathione conjugated to the *ade6* intermediate that was the precursor to the pigment, the hmt1p pump might be

the one being used for transportation into the vacuole. To determine if this was indeed so, we disrupted the *hmt1*$^+$ gene by the one-step PCR disruption method described in MATERIALS AND METHODS. The stable Ura$^+$ transformants obtained were screened for cadmium sensitivity and the cadmium-sensitive mutants were checked for the disruption at the *hmt1* locus by PCR (data not shown). One disruptant was obtained and we examined this *hmt1*Δ::*ura4*$^+$ *ade6-210* strain for pigmentation levels in adenine-limiting medium. There did not appear to be any significant decrease in pigmentation as compared to wild-type cells (Figure 2), suggesting that the hmt1p pump was not the transporter of the pigment precursor and that the pump was specific to cadystin peptides. This indicates that there must be a second pump specific for glutathione conjugates. A pump, specific for glutathione conjugates, has in fact been postulated (ORTIZ *et al.* 1995) based on *in vitro* experiments using isolated vacuoles, but it remains to be identified.

***YCF1*, a vacuolar glutathione-conjugate transporter, is required for pigment formation in *S. cerevisiae*:** The demonstration that pigment formation was sensitive to low concentrations of vanadate was strong, but still indirect, evidence that the pigment was being transported into the vacuole as a glutathione conjugate. The *YCF1* protein of *S. cerevisiae,* identified as a gene required for cadmium tolerance (SZYPYCZA *et al.* 1995), belongs to the family of ABC transporters with predominant sequence homology to the mammalian multidrug-related protein (MRP1) (COLE *et al.* 1992). Recent *in vitro* experiments carried out (LI *et al.* 1996) on *YCF1* indicated that the sequence homology extended to functional homology since the *YCF1* protein was demonstrated to transport, in an *in vitro* system, labeled dinitrophenyl-glutathione conjugates into isolated yeast vacuoles (LI *et al.* 1996). In fact, it was the major transporter of these conjugates in isolated vacuoles. Since our studies had indicated that the pigment precursor was being transported into the vacuoles as a glutathione-conjugate, we wished to examine pigment formation in *ade2* strains of *S. cerevisiae* bearing disruptions in *YCF1*. Strains of *S. cerevisiae* bearing disruptions in *YCF1* were constructed by the one-step PCR disruption method as described in MATERIALS AND METHODS. We found an 80–90% decrease in pigment formation in *ycf1*Δ strains as compared to *YCF1* strains as estimated spectrophotometrically (data not shown) and as seen on plates (Figure 6B). This clearly indicated that the adenine biosynthetic intermediates are indeed conjugated to glutathione followed by their transport into the vacuole primarily through specific glutathione-conjugate pumps.

DISCUSSION

The studies described in this report demonstrate conclusively, through genetic and molecular evidences, the involvement of glutathione in the formation of the intense red pigment in *ade6* mutants of fission yeast, a familiar, but little understood, phenotype to yeast researchers around the globe! In light of our findings, the partial structural analysis of the pigment carried out by SMIRNOV *et al.* in 1967 takes on a new meaning as it indicates a direct involvement of glutathione through the formation of a glutathione conjugate. The implications of the identification of significant levels of aspartic acid in addition to glutamic acid, glycine and cysteine in the pigment by these workers, however, is unclear. It is possible that aspartic acid may be conjugated to the molecule at some stage. An alternate possibility is that cysteic acid, which is generated from partial oxidation of cysteine during acid hydrolysis and migrates very close to aspartic acid on ion-exchange columns, might have been erroneously interpreted as aspartic acid. The formation of the glutathione conjugate receives further corroboration through our demonstration of the presence of a vanadate-sensitive pump for the transport of these conjugates into the vacuole, and from our demonstration that *YCF1,* a recently identified glutathione-conjugate transporter of *S. cerevisiae,* was the major pump transporting these compounds into the vacuole. Once inside the vacuole, it is likely that the more oxidizing environment of the vacuole and the secretory pathway may lead to the subsequent events of oxidative condensation and polymerization that lead to the characteristic pigmentation. Whether additional enzymes and factors are involved at this stage is also not clear.

The reason for conjugation of the intermediate to glutathione would appear to be the need for detoxification. In this context it is interesting to note that a study on *ade6* mutants of fission yeast observed that these cells accumulated a toxic metabolite that appeared to be inhibitory for the assembly of microtubules in both the nucleus and the cytoplasm (ISHIGURO 1989). It is probably to remove this unwanted product that the intermediate is conjugated to glutathione followed by its transport from the cytoplasm into the vacuole. Whether an intermediate phase I system that involves an oxidation step that precedes the conjugation step, as has been proposed (ISHIKAWA 1992), actually occurs in this case cannot be ascertained. However the clear demonstration of such a role for glutathione in yeast should allow the use of genetics to decipher these steps more rigorously. Our studies also indicate that the evolution of these pumps and pathways might have resulted more from the necessity to remove endogenously derived metabolites rather than for the detoxification of xenobiotics. In plants too, although a number of herbicides and other compounds are known to be detoxified by glutathione by pumping into the vacuole as a glutathione conjugate (MARTINOIA *et al.* 1993), endogenously derived metabolites have also been shown, very recently, to be conjugated to glutathione and transported to the vacuole (MAARS *et al.* 1995). The observa-

tion that γ-glutamylcysteine could also allow partial pigment formation (as seen in our analysis of the *gsh2Δ* strains) is also very interesting from the viewpoint of the evolution of this detoxification pathway, as it indicates that this intermediate can also be recognized by the enzymes and pumps that otherwise recognize glutathione. γ-glutamylcysteine could not replace, however, other functions of glutathione essential for growth, as the *gsh2Δ* cells were glutathione auxotrophs. These and many other features of this detoxification pathway require further elucidation and it is hoped that the demonstration of such a glutathione-mediated detoxification in yeasts would allow a more rigorous investigation into these events.

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